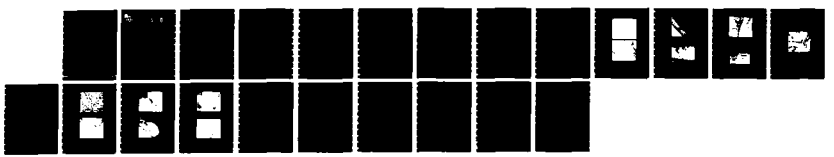
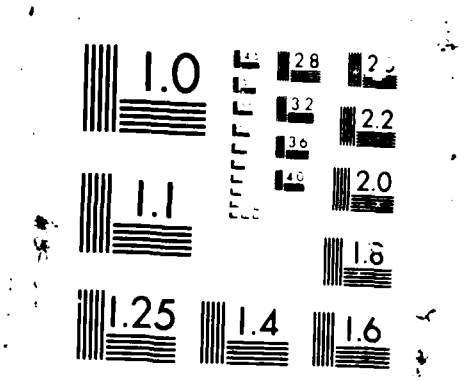
AD-A176 549 EFFECTS OF PHENYLDICHLORARSINE ON MACROPHAGES AND ENDOTHELIAL CELLS: A PR.. (U) LETTERMAN ARMY INST OF RESEARCH PRESIDIO OF SAN FRANCISCO CA

1/1

UNCLASSIFIED M P SARRAS ET AL. NOV 86 LAIR-228 F/G 15/2 NL




1.0
1.1
1.25
2.8
3.2
3.6
4.0
2.2
2.0
1.8
1.4
1.6

INSTITUTE REPORT NO. 228

EFFECTS OF PHENYLDICHLORARSINE ON MACROPHAGES AND ENDOTHELIAL CELLS: A PRELIMINARY ULTRASTRUCTURAL STUDY

Michael P. Sarras, Jr., PhD, MAJ, MSC[1,2]

Steven T. Schuschereba, MA[1]

and

John W. Harbell, PhD, CPT, MSC[1]

[1]Division of Comparative Medicine and Toxicology
Letterman Army Institute of Research

[2]Department of Anatomy
Division of Cell Biology
University of Kansas Medical Center
Kansas City, Kansas 66103

November 1986

LETTERMAN ARMY INSTITUTE OF RESEARCH
PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

Effects of phenyldichlorarsine on macrophages and endothelial cells: A preliminary ultrastructural study--Sarras et al.

This material has been reviewed by Letterman Army Institute of Research and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. *(AR 360-5)*

Edwin S Beattie 3 Dec 90

(Signature and date)

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Institute Report No. 228	2. GOVT ACCESSION NO. AD-A176549	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* Effects of Phenyldichlorarsine on Macrophages and Endothelial Cells: A Preliminary Ultrastructural Study.		5. TYPE OF REPORT & PERIOD COVERED Final, Aug 85- Nov 86. 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Michael P. Sarras, Jr., PhD., MAJ, MSC Steven T. Schuschereba, MA John W. Harbell, PhD., CPT, MSC		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Letterman Army Institute of Research Division of Pathology Presidio of San Francisco, CA 94129-6800		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Project #: 3A161101491C WU: 064
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Command Fort Detrich, Frederick, MD 21701		12. REPORT DATE November 1986 13. NUMBER OF PAGES 16
14. MONITORING AGENCY NAME & ADDRESS*(if different from Controlling Office)*		15. SECURITY CLASS. *(of this report)* Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*
This document has been approved for public release and sales; its distribution is unlimited.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*
Phenyldichlorarsine, RAW Mouse macrophage, Human vascular endothelium, Cell culture, vacuole;

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*
The cellular mechanisms involved in arsenical-induced vesication are unknown. To approach this problem, tissue culture studies were undertaken to correlate morphological alterations with previously reported changes in cell growth that are induced by phenyldichlorarsine (PDA), a monosubstituted trivalent arsenical. Changes in the morphology of human capillary endothelial cell cultures and RAW 264.7, an established line of mouse macrophage cells, were monitored by scanning and transmission electron micro-

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

#20

scopy. The results of this study, indicate that over a broad range of concentrations, PDA induces morphological changes in both mouse macrophages and human endothelial cells. At doses of PDA which cause a 50% reduction in cell growth rate (30 nM), ultrastructural changes include a) an increase in large cytoplasmic vacuoles in macrophages and b) the appearance of autophagic vacuoles in endothelial cells. At higher PDA concentrations (≥60 nM), ultrastructural changes include a) loss of large cytoplasmic vacuoles in macrophages as well as a general rounding of the cell and a loss of the normal surface membrane projections and b) the appearance of cell surface blebbing in human endothelial cells. The observed morphological alterations indicate that, even at low concentration, PDA can affect cellular processes.

Effects of Phenyldichlorarsine on Cultured Cells

ABSTRACT

The cellular mechanisms involved in arsenical-induced vesication are unknown. To approach this problem, tissue culture studies were undertaken to correlate morphological alterations with previously reported changes in cell growth that are induced by phenyldichlorarsine (PDA), a monosubstituted trivalent arsenical. Changes in the morphology of human capillary endothelial cell cultures and RAW 264.7, an established line of mouse macrophage cells, were monitored by scanning and transmission electron microscopy. The results of this study indicate that over a broad range of concentrations, PDA induces morphological changes in both mouse macrophages and human endothelial cells. At doses of PDA which cause a 50% reduction in cell growth rate (30 nM), ultrastructural changes include a) an increase in large cytoplasmic vacuoles in macrophages and b) the appearance of autophagic vacuoles in endothelial cells. At higher PDA concentrations (≥60 nM), ultrastructural changes include a) loss of large cytoplasmic vacuoles in macrophages as well as a general rounding of the cell and a loss of the normal surface membrane projections and b) the appearance of cell surface blebbing in human endothelial cells. The observed morphological alterations indicate that, even at low concentrations, PDA can affect cellular processes.

Effects of Phenyldichlorarsine on Cultured Cells

INTRODUCTION

Since the introduction of organic arsenicals by the Germans in World War I (1), there has been military medical interest in these compounds. Despite extensive study, the cellular mechanisms of trivalent arsenicals involved in vesication remain unknown. Alterations in fluid balance and subsequent inflammatory responses in affected skin areas during vesication suggest the involvement of components of the circulatory and immune systems. The importance of capillary endothelial cells in fluid and solute transport by blood vessels and of tissue macrophages in the inflammatory process makes both of these cells potential sites of dysfunction during the vesication process. Arsenical action on these cell types is best monitored under in vitro conditions where all parameters can be controlled. The present study was therefore designed to evaluate the in vitro effects of one organic arsenical, phenyldichlorarsine (PDA), on the morphology of human capillary endothelial cell cultures and a transformed mouse macrophage cell line (RAW 264.7).

MATERIALS AND METHODS

Cells (both endothelial and macrophage lines) were cultured in Falcon 3006 optical tissue culture dishes (60 x 15 mm) with removable liners. Macrophage cells (RAW 254.7) (2) were cultured in GEM 1717 media with 10% fetal calf serum (FCS), and endothelial cells (originally isolated from omental fat capillaries - 4th passage) (3) were cultured in MCDB 131 with 4% dialyzed FCS. For experimental treatment, cultures were exposed to increasing concentrations of PDA (10, 20, 30, 40, 60, 80, 100, and 120 nM) in 5 ml of medium per dish. Controls were cultured without PDA. For experiments involving low doses of PDA (10 to 40 nM), endothelial cells were plated at approximately 10^6 cells per dish, while RAW 264.7 cells were plated at 6×10^5 cells per dish. PDA concentration (M) per cell for the RAW and endothelial cells is presented in Table I. In such experiments, cultures were confluent at the time PDA was added to the dishes. For experiments involving high doses of PDA (≥40 nM), cells were plated at a lower density and cultures were not confluent when PDA was added to the dishes. In all experiments, cells were exposed to PDA for 13 hours prior to fixation. Immediately after PDA treatment, cells were immersed for 2 hours in Karnovsky's fixative (diluted 1:4.5 with distilled water and 0.2 M Na cacodylate to give 0.73% paraformaldehyde, 0.91% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4) at 24°C (4). Following fixation, specimens were washed with 0.1 M Na cacodylate

buffer, pH 7.4, and postfixed at 4°C for 1 hour in 1% OsO_4 buffered with 0.1 M Na cacodylate (pH 7.4). Culture liners with cells attached were dehydrated through a graded ethanol series and then were cut with razor blades into 10- to 20-mm^2 areas for continued processing.

TABLE I PDA CONCENTRATION PER CELL

Concentration of PDA given nM	RAW Cells(10^6) M/cell	Endothelial Cells ($6X10^5$) M/cell
10	5.00×10^{-17}	8.33×10^{-17}
20	1.00×10^{-16}	1.67×10^{-17}
30	1.50×10^{-16}	2.50×10^{-16}
40	2.00×10^{-16}	3.33×10^{-16}
60	3.00×10^{-16}	5.00×10^{-16}
80	4.00×10^{-16}	6.67×10^{-16}
100	5.00×10^{-16}	8.33×10^{-16}
120	6.00×10^{-16}	1.00×10^{-15}

Effects of Phenyldichlorarsine on Cultured Cells

For transmission electron microscopy (TEM), specimens were transferred into Spurr's embedding medium (5), and after 2 hours at 24°C, new Spurr's was added. Following an additional 2 hours in Spurr's, specimens were cut into 2- to 5-mm^2 sections, placed in embedding molds, and after an additional 12 hours at 24°C, were polymerized at 60°C. Thick sections were cut with glass knives and stained with methylene blue (6). Thin sections were cut with a diamond knife and stained with 2% uranyl acetate in 50% ethanol and with lead citrate (7). Specimens were examined and photographed with a Philips 201 transmission electron microscope at 60 kV.

For scanning electron microscopy (SEM), specimens were critical point dried using a Denton DCP-1 apparatus (Denton Vacuum, Inc., Cherry Hill, NJ) and then sequentially coated with carbon, silver, and gold (≤15 nm total thickness) using a Denton DV-502 vacuum evaporator (Denton Vacuum, Inc., Cherry Hill, NJ) with a specimen rotary attachment. Specimens were examined and photographed using an ETEC Autoscan scanning electron microscope at 10 kV.

RESULTS

A. Endothelial cell analysis

In vitro exposure of endothelial cell cultures to PDA induced morphological changes detectable by both SEM and TEM. As viewed by SEM, untreated human endothelial cells were squamous in morphology with cell-cell contacts (Fig. 1a). Cells displayed a complex apical surface morphology which contained numerous microvilli (Fig. 1b). TEM analysis indicated that cell-cell contacts appeared to involve the formation of tight junctions (Fig. 2). Additional TEM analysis indicated a central nucleus with a normal complement of cytoplasmic organelles including free polysomes, rough endoplasmic reticulum (RER), microfilaments, Golgi, mitochondria, and numerous vesicular structures (Fig. 3). The cultured endothelial cells also contained apical and basal coated pits with associated caveolae similar to those observed during in vivo studies (8) (Fig. 4). TEM analysis indicated that low levels of PDA (30 nM) induced the autophagic vacuoles (Fig. 5). At higher concentrations of PDA (≥60 nM), SEM analysis indicated that plasma membrane blebbing was induced (Fig. 6). The altered surface morphology was observed as spherical evaginations, 1-8 μm in diameter, which occurred in single or clustered arrays (Fig. 6).

Effects of Phenyldichlorarsine on Cultured Cells

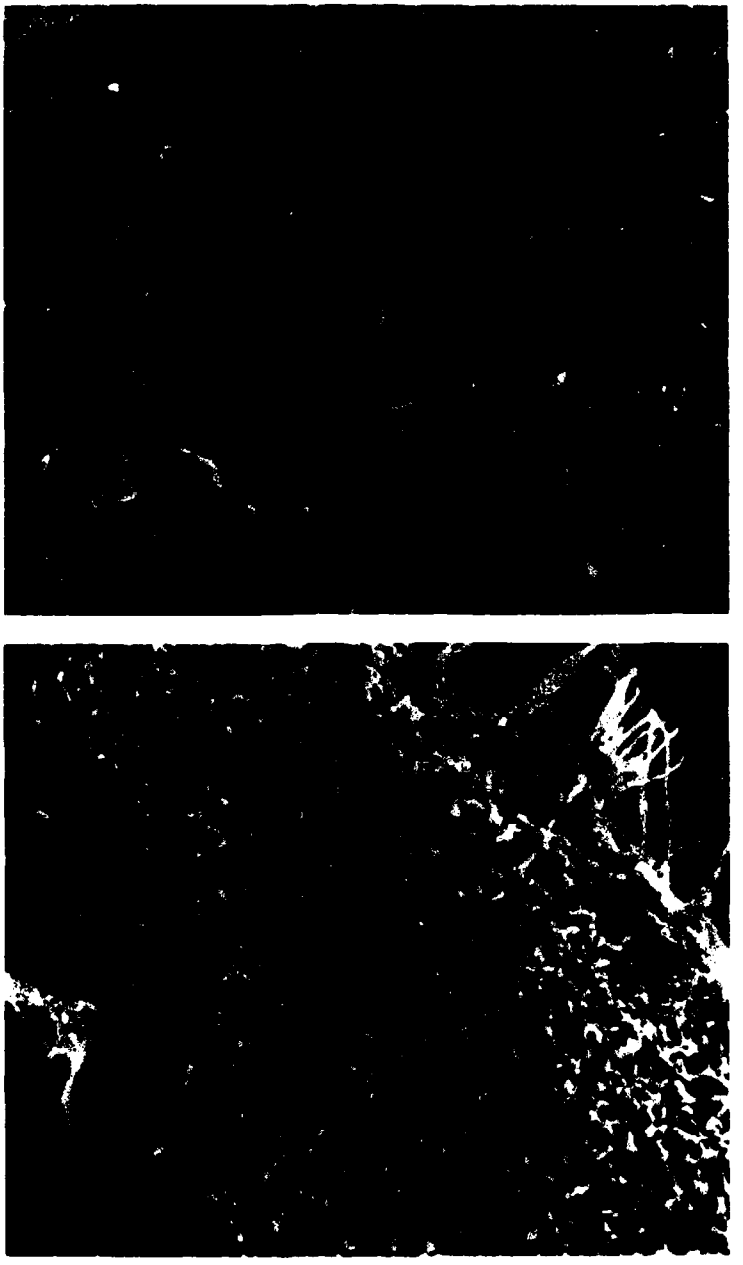


Figure 1. Scanning electron micrograph of a culture of human endothelial cells. As shown in Fig. 1a, cells are squamous in shape with cell-cell contacts (arrowheads). At higher magnifications (Fig. 1b) the apical surface appears complex in nature with numerous microvilli. Magnification: 1a, 202X; 1b, 2025X.

Effects of Phenyldichlorarsine on Cultured Cells

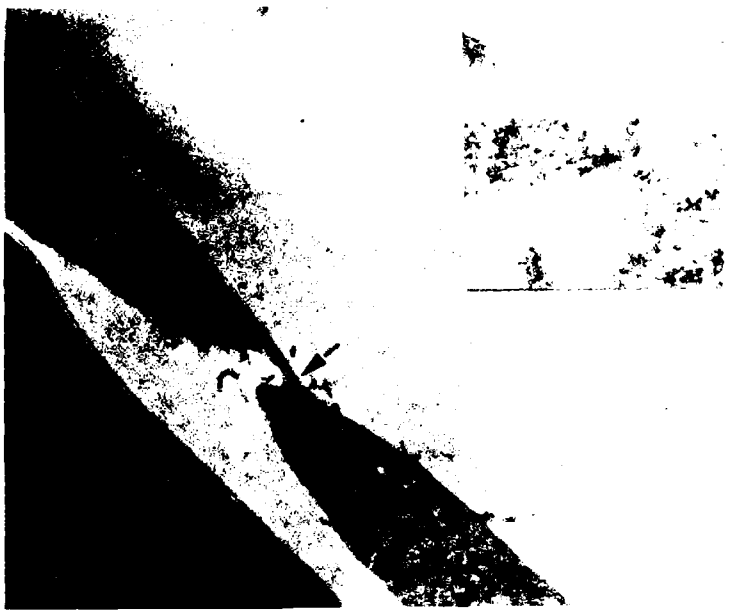


Figure 2. Transmission electron micrograph of human endothelial cell-cell contact regions which appear as tight junctional complexes (arrow). The insert shows the region indicated by the arrow at higher magnification. Magnification: 6,075X; insert, 40,500X.

Figure 3. Transmission electron micrograph of a human endothelial cell. Cell contains a normal range of organelles such as rough endoplasmic reticulum (small arrow), microfilaments (large arrow), and surface microvilli (arrowhead). Magnification: 23,800X.

Effects of Phenyldichlorarsine on Cultured Cells

Figure 4. Transmission electron micrograph of an endocytic pit of a plasma membrane with associated caveolae (insert) in a human endothelial cell. Magnification: 94,500X; insert, 119,000X.

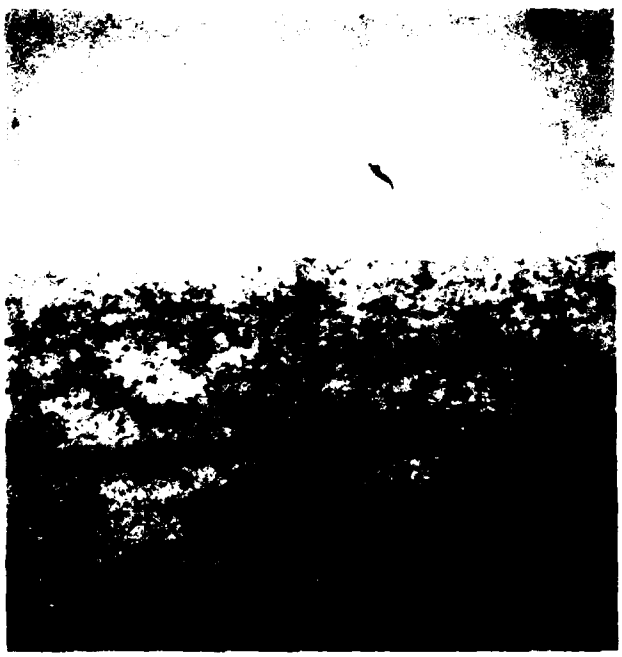


Figure 5. Transmission electron micrograph of an autophagic vacuole (secondary lysosome) (arrowhead) induced in a human endothelial cell by exposure to 30 nM PDA. Magnification 40,500X.

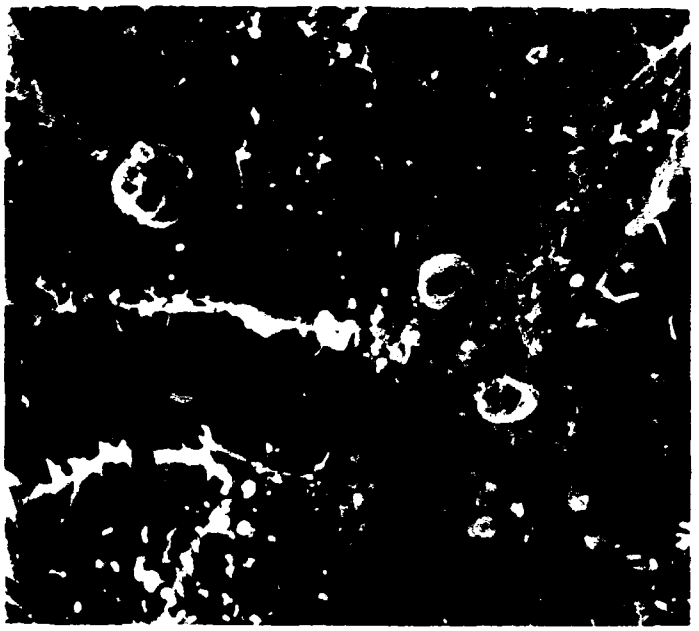


Figure 6. Scanning electron micrograph of surface alterations induced in a human endothelial cell by exposure to 120 nM PDA. Surface alteration included the appearance of large (arrow) and small (arrowheads) surface blebs. Magnification: 2,025X.

B. Macrophage analysis

In vitro exposure of macrophage cultures to PDA induced morphological changes detectable by both SEM and TEM. As viewed by SEM, untreated macrophage cells appeared heterogeneous in morphology. While most cells were spherical, a subpopulation appeared fusiform and fibroblast-like (Fig. 7). Surface morphology appeared complex with numerous spindle-like and ruffled-border plasma membrane projections (Fig. 8). As with endothelial cells, TEM analysis indicated a normal complement of cellular organelles including rough endoplasmic reticulum, free polysomes, Golgi, microfilaments, microtubules, mitochondria, numerous vesicles, and large cytoplasmic vacuoles which contained amorphous material and virus particles (Type C) (2), which are commonly shed by these cells (Fig. 9). At low PDA concentrations (10 to 30 nM), TEM analysis indicated an apparent increase in the number of large cytoplasmic vacuoles (Fig. 10). At higher PDA concentrations (120 nM), TEM analysis indicated a loss of these cytoplasmic vacuoles and a general rounding of the entire cell with an accompanying loss of the cell's normal surface membrane projections (Fig. 11). Subsequent analysis by SEM confirmed these TEM observations and indicated that at higher PDA concentrations (60-120 nM) cells were more rounded in appearance and had a more simplified surface morphology with fewer spindle-like and ruffled-border membrane projections (Fig. 12).

DISCUSSION

Because of ethical considerations, investigators use animal models and cell culture procedures to study the mechanisms by which arsenicals induce vesiculation in mammalian systems. The experiments described in the present study utilized human endothelial and RAW 264.7 cell cultures to evaluate morphological changes induced by PDA, a monosubstituted trivalent arsenical. Endothelial cells were chosen because of their possible involvement in the vesiculation process, which is characterized by alterations in fluid balance (a circulatory-system mediated event which involves endothelial cells). RAW 264.7 cells were examined because of their involvement in subsequent inflammatory processes.

Previous studies have shown that PDA has approximately the same vesicant potency and systemic toxicity as other arsenicals such as lewisite (1). These findings have been elaborated by McGown et al. (9) who have observed

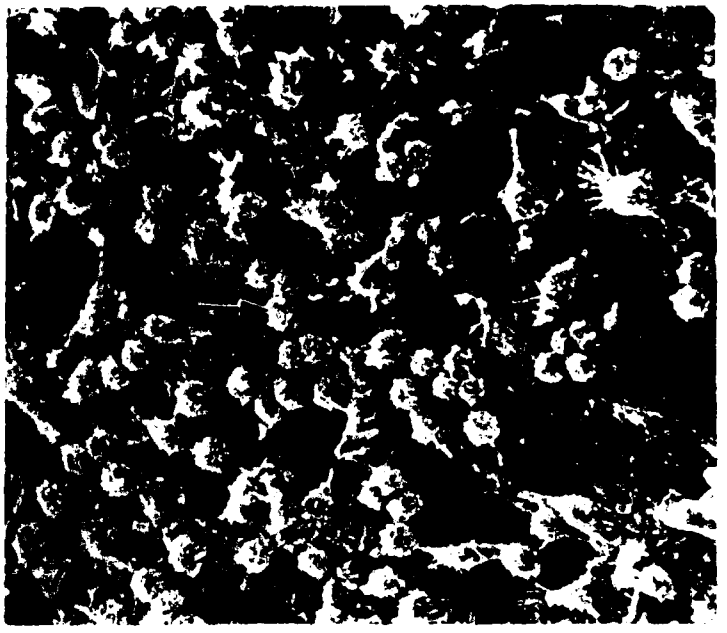


Figure 7. Scanning electron micrograph of untreated mouse macrophages (Raw 264.7). As shown, cells are heterogeneous in structure. While most cells appear rounded (arrow), some are fusiform (arrowhead). Magnification: 405X.

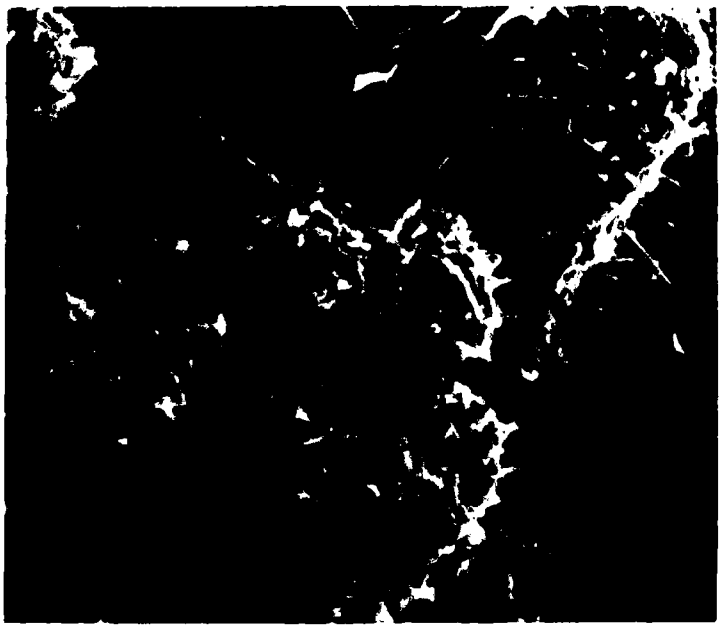


Figure 8. Scanning electron micrograph of untreated cells from the macrophage culture shown in Fig. 7. Cells display a complex surface morphology with numerous microvilli and ruffled-border plasma projections. Magnification: 2,025X.

Figure 9. Transmission electron micrograph of untreated macrophages. Cells contained a normal complement of organelle systems as well as large vacuoles containing virus particles (arrow). Magnification: 9,450X.

Figure 10. Transmission electron micrograph of a mouse macrophage exposed to 30 nM PDA. As compared to controls, an apparent increase in the number of large cytoplasmic vacuoles containing virus particles was observed. Magnification: 9,450X.

Effects of Phenyldichlorarsine on Cultured Cells

Figure 11. Transmission electron micrograph of a mouse macrophage exposed to 120 nM PDA. As indicated, PDA exposure induced a general rounding of the entire cell with an accompanying loss of the cell's normal surface membrane projections. In addition, cytoplasmic vacuoles containing virus particles were absent. Magnification: 14,000X.

Figure 12. Scanning electron micrograph of a mouse macrophage exposed to 120 nM PDA. As indicated in transmission micrographs (Fig. 11), scanning images indicated a general rounding of the cell with a retraction of surface membrane projections. Magnification: 4,050X.

extensive dermatological alterations in human skin-grafted athymic nude mice treated topically with PDA. In addition, McGown et al. (10) have reported a 50% inhibition of growth in L5178Y mouse lymphoma cells and RAW 264.7 mouse macrophages when exposed for either 1 or 24 hours to 30-50 nM PDA. Based on these previous in vitro studies, the present study analyzed morphological alterations induced in cells exposed to 10-120 nM PDA for 13 hours.

At concentrations as low as 30 nM, PDA induced ultrastructural changes in both RAW 264.7 cells and endothelial cells. In macrophages, changes involved the formation of large cytoplasmic vacuoles. The origin and function of the vacuoles could not be determined from these morphological studies. Previous studies have reported that these cells exhibit most of the differentiated characteristics of normal macrophages and are known to be actively phagocytic (10). Whether the observed membrane-bound cytoplasmic vacuoles are phagocytic in nature or originate from the Golgi remains to be determined. The presence of virus particles in these cytoplasmic vacuoles suggests that they are phagocytic in nature since enveloped viruses are normally not assembled until protein and nucleic acid components are transported to the plasma membrane (11). The loss of these cytoplasmic vacuoles at higher PDA concentrations may represent decreased phagocytic function of the cells due to the toxic effects of the compound. This concept is supported by the observation that the apparent loss of cytoplasmic vacuoles correlates with a general rounding of cells and a loss of cell surface projections. Cell rounding is associated with a general loss of cell function and viability under various toxic conditions (12). The physiological basis for these changes awaits confirmation by additional ultrastructural studies involving pulse-chase experiments using such membrane markers as cationized ferritin.

In the case of endothelial cells, low concentrations of PDA (30 nM) induced the formation of autophagic vacuoles (secondary lysosomes). The presence of other cytoplasmic organelles, such as membrane fragments and ribosomes, in these vacuoles, confirms the classification of these vacuoles as autophagic in nature and suggests the involvement of lysosomal and endocytic processes. The presence of coated pits and associated caveolae in the endothelial cells makes these cells well suited for the study of endocytic and related events. If the vacuoles observed in the macrophage cells were phagocytic in nature, then the presence of autophagic

vacuoles in endothelial cells suggests that low PDA concentrations induce stimulation of lysosomal and endocytic activity in two completely different cell types. The influence of PDA on endocytic- and lysosomal-type cellular processes therefore requires future study since the latter process could have an important role during vesiculation. In contrast to RAW 264.7 cells, high concentrations of PDA (120 nM) induce cell surface blebbing in endothelial cells. The significance of these membrane changes in endothelial cells is unclear but, as with macrophages, may reflect altered cell function and decreased cell viability. To better evaluate this point, direct testing for cell viability is required using dye exclusion methods (13).

Acknowledgments

The authors wish to thank Drs. Phillip D. Bowman, Evelyn L. McGown, and Paul W. Mellick for scientific reviews, Antonio A. Somera for technical assistance, and Ms. Brenda Tennyson-Jackson and Mrs. Dianna Johnson for their help in preparation of this manuscript. The publication cost of this study was supported in part by NIH Grant AM33362, awarded to MPS.

REFERENCES

1. Gates M, Williams JW, Zapp A. Arsenicals. In: Chemical warfare agents and related chemical problems. Summary Technical Report of Division 9, NDRC, Volume 1, 1946.

2. Raschke WC, Baird S, Ralph P, Nikoih I. Functional macrophage cell line transformed by Abelson leukemia virus. Cell 1978;15:261-267.

3. Kern PA, Knedler A, Eckel RH. Isolation and culture of microvascular endothelium from human adipose tissue. J Clin Invest 1983;71:1822-1829.

4. Karnovsky MJ. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 1965;27:137A-138A.

5. Spurr AR. A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 1969;26:31-43.

6. Huber JD, Parker F, Odland GF. A basic fuschin and alkalinized methylene blue rapid stain for epoxy-embedded tissue. Stain Technol 1968;43:83-87.

7. Venable JH, Coggeshall R. A simplified lead citrate stain for use in electron microscopy. J Cell Biol 1965;25:407-408.

8. Burns RR, Palade GE. Studies on blood capillaries. J Cell Biol 1968;37:244-276.

9. McGown EL, van Ravensway T, Dumlao CR, Black CE, Mellick PW. Histological changes caused by application of lewisite analogs to mouse skin and human skin xenographs. In: USMRDC Fifth Annual Chemical Defense Bioscience Review. May:1985, p.96.

10. McGown EL, Harbell JW, Dumlao CR, O'Connor RJ. Use of in vitro cell systems to study cytotoxic effects of vesicant arsenicals and efficacy of antidotes. In: USAMRDC Fifth Annual Chemical Defense Bioscience Review. May:1985, p.291-301.

11. Rodriguez-Boulan E. Membrane biogenesis, enveloped RNA viruses, and epithelial polarity. In: Satir BH, ed. Modern cell biology; volume 1. New York:Raven Press 1983; 119-170.

12. Nicolson GL. Transmembrane control of the receptors on normal and tumor cells: I. Cytoplasmic influence over cell surface components. Biochim Biophys Acta 1976;457:57-108.

13. Freshney RI. Culture of animal cells. New York: Alan R. Liss, 1983.

OFFICIAL DISTRIBUTION LIST

Commander
US Army Medical Research
and Development Command
ATTN: SGRD-RMS/Mrs. Madigan
Fort Detrick, MD 21701-5012

Defense Technical Information Center
ATTN: DTIC/DDAB (2 copies)
Cameron Station
Alexandria, VA 22304-6145

Office of Under Secretary of Defense
Research and Engineering
ATTN: R&AT (E&LS), Room 3D129
The Pentagon
Washington, DC 20301-3080

The Surgeon General
ATTN: DASG-TLO
Washington, DC 20310

HQ DA (DASG-ZXA)
WASH DC 20310-2300

Commandant
Academy of Health Sciences
US Army
ATTN: HSHA-CDM
Fort Sam Houston, TX 78234-6100

Uniformed Services University
of Health Sciences
Office of Grants Management
4301 Jones Bridge Road
Bethesda, MD 20814-4799

US Army Research Office
ATTN: Chemical and Biological
Sciences Division
PO Box 12211
Research Triangle Park, NC 27709-2211

Director
ATTN: SGRD-UWZ-L
Walter Reed Army Institute
of Research
Washington, DC 20307-5100

Commander
US Army Medical Research Institute
of Infectious Diseases
ATTN: SGRD-ULZ-A
Fort Detrick, MD 21701-5011

Commander
US Army Medical Bioengineering
Research & Development Laboratory
ATTN: SGRD-UBG-M
Fort Detrick, Bldg 568
Frederick, MD 21701-5010

Commander
US Army Medical Bioengineering
Research & Development Laboratory
ATTN: Library
Fort Detrick, Bldg 568
Frederick, MD 21701-5010

Commander
US Army Research Institute
of Environmental Medicine
ATTN: SGRD-UE-RSA
Kansas Street
Natick, MA 01760-5007

Commander
US Army Institute of Surgical Research
Fort Sam Houston, TX 78234-6200

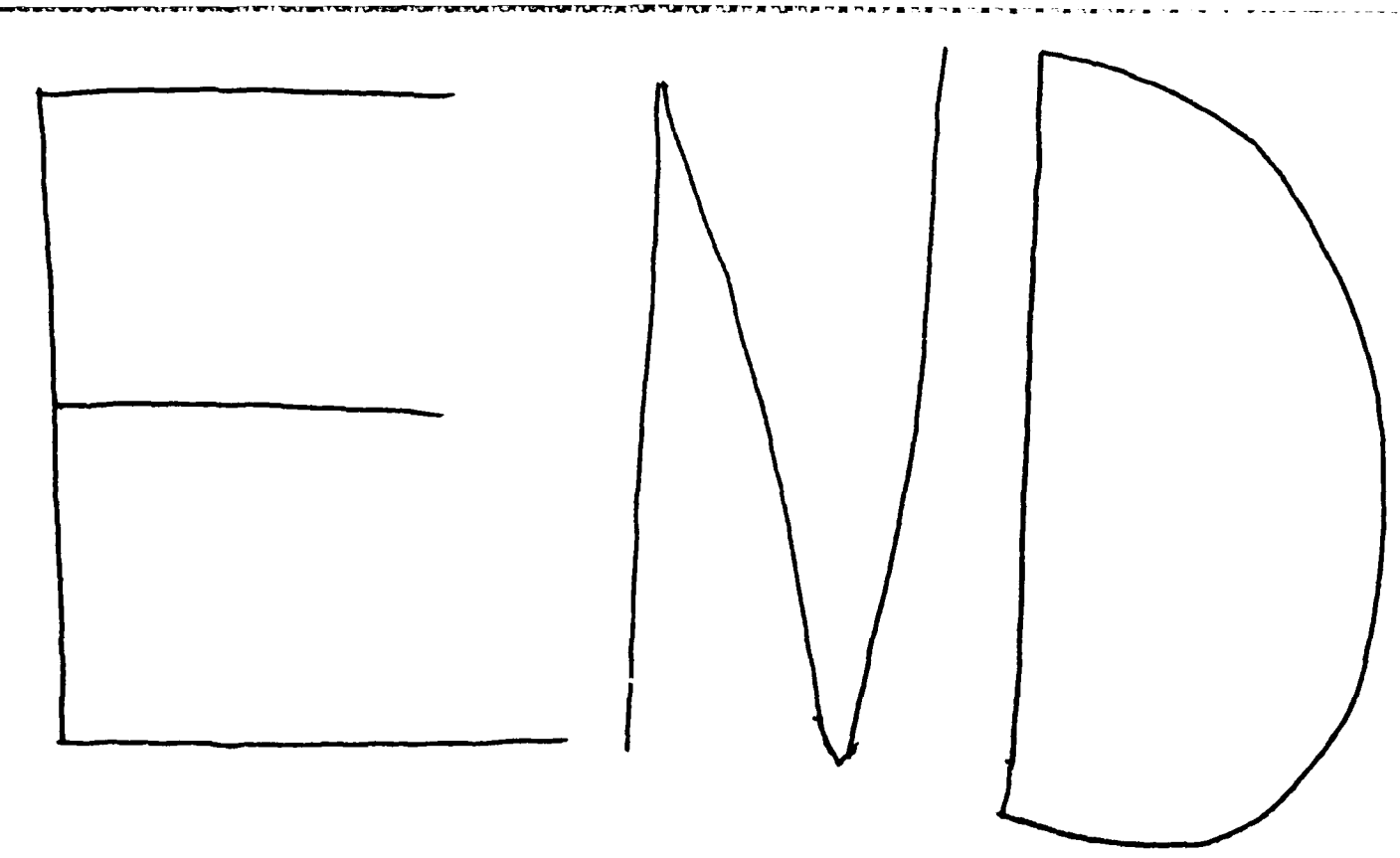
Commander
US Army Research Institute
of Chemical Defense
ATTN: SGRD-UV-AJ
Aberdeen Proving Ground, MD 21010-5425

Commander
US Army Aeromedical Research Laboratory
Fort Rucker, AL 36362-5000

AIR FORCE Office of Scientific
Research (NL)
Building 410, Room A217
Bolling Air Force Base, DC 20332-6448

Commander
USAFSAM/TSZ
Brooks Air Force Base, TX 78235-5000

Head, Biological Sciences Division
OFFICE OF NAVAL RESEARCH
800 North Quincy Street
Arlington, VA 22217-5000



3-87

DTIC